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(21) International Application Number: PCT/US93/04582 (22) International Filing Date: 13 May 1993 (13.05.93) (30) Priority data: 07/882,478 13 May 1992 (13.05.92) US (71) Applicant (for all designated States except US): THE BETH ISRAEL HOSPITAL ASSOCIATION [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US). (72) Inventor; and (75) Inventor/Applicant (for US only) : FOSSEL, Eric, T. [US/US]; 66 Priscilla Road, Chestnut Hill, MA (US). (74) Agent: LORUSSO & LOUD; 440 Commercial Street, Boston, MA 02109 (US).		(81) Designated States: AT, AU, BB, BG, BR, CA, CH, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.
(54) Title: TARGETED ACTIVATED SPECIES CYTOTOXICITY (57) Abstract This invention comprises a method of treating animals, including humans, for conditions such as cancer by producing discrete site cytotoxic environment in an animal, including a human, by the steps of administering to the animal a therapeutically effective dosage of a prooxygenator-affixation element complex; in conjunction with, administering to the animal a therapeutically effective amount of an oxygen source substrate thus producing oxygen free radical species including superoxide at the site of binding. The present invention further comprises a prooxygenator-affixation element complex. In one embodiment the prooxygenator aspect is xanthine oxidase, the affixation element is a tumor specific antibody and the oxygen source substrate is xanthine.		

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1 TARGETED ACTIVATED SPECIES CYTOTOXICITY
23 Field of the Invention.
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5 This invention comprises a method of treating animals,
6 including humans, for conditions such as cancer by producing a
7 discrete site cytotoxic environment in an animal, including a
8 human, by the steps of administering to an animal a
9 therapeutically effective dosage of a proöxygenator-affixation
10 element complex; in conjunction with, administering to the animal
11 a therapeutically effective amount of an oxygen source substrate
12 thus producing oxygen free radical species including superoxides
13 at a cytotoxic level at the site of complex binding. The present
14 invention further comprises a proöxygenator-affixation element
15 complex. In one embodiment the proöxygenator moiety is xanthine
16 oxidase, the affixation element is a tumor specific antibody and
17 the oxygen source substrate is xanthine.

18 Background of the Invention.

19 The earliest medicinal agents were administered either
20 typically or by ingestion with little control over the site of
21 drug action. The discovery of penicillin brought the "magic
22 bullet" to the practice of medicine. Since then pharmacology has
23 continued to refine techniques to bring the active agents into
24 the closest proximity with the site of action. For example,
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1 today, radio labeled antibodies are used to localize sites in
2 diagnostic procedures. Similarly, IL-2 binding sites have been
3 linked to diphtheria toxin to target and destroy activated
4 T-cells. However, this last approach has been limited to cell by
5 cell killing of those cells which actually phagocytize the
6 diphtheria toxin molecule.

7 A major problem with chemotherapy is toxicity.
8 Chemotherapeutic agents are characterized by high toxicity. This
9 toxicity is only slightly discriminatory, and, as a general
10 principal, attacks the entire body injuring or destroying both
11 normal and abnormal tissue. Many solid tumors are well
12 vascularized. However cellular antitumor agents have difficulty
13 reaching tumor cells, in part, due to a fibrin barrier. In
14 certain instances, the fibrin barrier may be eliminated by
15 thrombolytic agents. In other instances, treatment of cancers,
16 and particularly solid tumors, is hampered by inadequate
17 circulatory investment of such tumors. In fact, the most rapidly
18 growing tumors may be the most difficult ones in which to obtain
19 therapeutic concentrations of anti-tumor agents.

20 The present invention is, in its preferred embodiment,
21 directed to providing the delivery of one or more therapeutic
22 agents quite specifically to a given site, but not so
23 specifically that only a given individual cell is treated. The
24 therapeutic agents are activated oxygen species (collectively,
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1 "AOS"). AOS of the present invention include peroxides such as
2 hydrogen peroxide (H_2O_2) and superoxide (O_2^-), and singlet oxygen
3 (1O_2). It is just such AOS that have been conjectured as active
4 agents in polymorphonuclear neutrophils after activation by
5 pathogens, cytokines or other cell activators.

6 This invention utilizes the known technology of binding
7 compounds to antibodies so that neither the ability of the
8 antibody to bind to antigen nor the activity of the bound
9 compound is impaired. An examples of this technology are U.S.
10 Pat. No. 4,671,958 issued to Rodwell et al., and U.S. Pat. No.
11 4,867,973 to Goers et al, the teachings of each being
12 incorporated herein by reference. U.S. Pat. No. 4,671,958
13 describes a method for site specific covalent attachment of a
14 compound to an antibody molecule by selectively oxidizing a
15 carbohydrate moiety of the antibody, located outside the antigen
16 binding region of the antibody, to form an aldehyde group with an
17 amine group (such as a primary amine, secondary amine, hydrazine,
18 hydrazide, hydroxylamine, phenylhydrazine or semicarbazide) to
19 form a Schiff base (e.g., oxime, hydrazone, phenylhydrazone, or
20 semicarbazone, respectively).

21 Accordingly, substrate linkers are modified by attaching
22 hydrazine or hydrazide derivatives to one end of the linker. The
23 unmodified sites on the linker may or may not be covalently
24 attached to a compound. Linkers are synthetic or naturally
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1 occurring substrates which are susceptible to cleavage by any of
2 the components of complement. A number of such linkers are
3 described and disclosed in U.S. Pat. No. 4,671,958, including
4 N-Boc-tyrosine o-nitrophenyl ester, N-acetyl-gly-lys-methyl ester
5 and others well known in the art.

6 By way of example, substrate linkers which are attached to a
7 compound via an ester or amide link, are modified by attaching a
8 hydrazide such as phenylhydrazine to the opposite amino terminus
9 of the peptide chain. The hydrazide derivative of the peptide
10 linker is attached to a compound via an ester or amide link is
11 then reacted with an oxidized immunoglobulin fragment containing
12 an oxidized carbohydrate. This results in hydrazone formation
13 and the covalent attachment of the compound to the carbohydrate
14 side chain of the immunoglobulin via a linker group which is
15 susceptible to cleavage by complement. The described covalent
16 attachment of linker to the carrier antibody does not interfere
17 with the antigen binding site of the molecule nor with complement
18 fixation. Schematically this may be represented:



21 where β represents an amide or ester bond.

22 Summary of the Invention.

23 This invention includes a proöxygenator-affixation element
24 complex. In particular embodiments the proöxygenator-affixation
25 element complex comprises a proöxygenator moiety of at least one

1 enzyme, such as xanthine oxidase, superoxide dismutase, or a
2 myeloperoxidase. In specific embodiments of the invention the
3 proöxygenator-affixation element complex comprises an affixation
4 element being an antibody. Particular antibodies are those that
5 bind to melanoma, carcinoma, adenocarcinoma, sarcoma,
6 neuroblastoma, myeloma, lymphoma, or leukemia cells. Examples
7 within the invention are antibodies such as α -MSH,
8 carcino-embryonic antigen, α -fetoprotein, or SSEA-1. Other
9 examples are wherein proöxygenator-affixation element complex
10 comprises an affixation element being an peptide such as the
11 diphtheria fragment B, or IL-2 binding site.

12 This invention also includes a method of producing discrete
13 site cytotoxic environment in an animal, including a human,
14 comprising the steps of:

15 (i) administering to said animal a therapeutically effective
16 dosage of a proöxygenator-affixation element complex wherein said
17 complex has a binding affinity for the site of cytotoxic
18 environment production; and thereafter,

19 (ii) administering to said animal a therapeutically
20 effective amount of an oxygen source substrate. In a particular
21 embodiment, upon administration, the affixation element of the
22 proöxygenator-affixation element complex performs the step of
23 binding the complex to a cell. Some proöxygenator elements of
24 the method are xanthine oxidase, superoxide dismutase, or
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1 myeloperoxidase. Certain oxygen source substrates of the method
2 are methylxanthines such as xanthine, caffeine or theophylline.
3 The method of this invention also encompasses the step of
4 maintaining the AOS concentration in a discrete area to at least
5 about 10^{-8} M/minute for a particular intervals of at least about
6 15 minutes, and preferably at least about 10^{-6} M/minute for a
7 particular interval or intervals of at least about 15 minutes,
8 and more preferably at least about 10^{-5} M/minute for a
9 particular intervals of at least about 15 minutes. In particular
10 aspects the method includes administering to an animal a
11 therapeutically effective dosage of a proöxygenator-affixation
12 element complex which comprises binding said complex to at least
13 about 50% and preferably at least about 80% of the binding sites
14 at the general site of cytotoxic environment production.

15 In a diagnostic application, this invention includes adding
16 to a tissue culture of a tumor to be tested two or more graduated
17 dosages of a proöxygenator-affixation element complex wherein
18 said complex has a binding affinity for the tumor being tested;
19 and thereafter,

20 administering to said culture a therapeutically effective
21 amount of an oxygen source substrate;

22 determining tumor growth inhibition in said tissue culture.

23 Detailed Description of the Invention.
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1 This invention will best be understood with reference to the
2 following definitions:

3 A. Proöxygenator shall mean at least one moiety which
4 produces an AOS upon exposure to at least one oxygen bearing
5 substrate.

6 In some applications it will be appreciated that multiple
7 proöxygenator moieties may be attached to a single affixation
8 moiety. These may be the same proöxygenator moieties or
9 different proöxygenator moieties. In one example, xanthine
10 oxidase and a peroxidase such as superoxide dismutase may be
11 cojoined to a single affixation moiety. Additionally, marker
12 moieties such as radio labels, fluorescent materials or NMR
13 labels may be affixed.

14 B. AOS of the present invention shall mean activated oxygen
15 species including peroxides such as hydrogen peroxide (H_2O_2) and
16 oxygen free radicals, ($O_2^{\cdot-}$), HO^{\cdot} , and HOO^{\cdot} . The particular AOS,
17 $O_2^{\cdot-}$, is termed "superoxide."

18 AOS of this invention further include singlet oxygen (1O_2).

19 Paradigm reactions of this invention are (1) the conversion
20 of xanthine to superoxide, the oxygen free radical ($O_2^{\cdot-}$) by the
21 enzyme xanthine oxidase, and (2) the conversion of xanthine to
22 uric acid and superoxide, an oxygen free radical ($O_2^{\cdot-}$).
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1 Without being bound to a particular theory, it is believed
2 that the efficacy of this invention is a consequence of the
3 provision of AOS in therapeutically effective concentrations to
4 the site of complex binding. While not bound by any particular
5 scheme by which the provision of AOS to the site of complex
6 binding provide therapeutic efficacy, it is believed that the
7 desired reaction such as tumor toxicity is substantially similar
8 to the cytotoxic and bactericidal system found in
9 polymorphoneuclear neutrophil leukocyte (PMN). In PMN systems,
10 researchers have found that ($O_2^{\cdot-}$), HO^{\cdot} , and HOO^{\cdot} are directly
11 cytotoxic. In addition H_2O_2 may react with Cl^- to form OCl^-
12 (hypochlorite ion) which is a bactericidal agent. In addition to
13 hydrogen peroxide, the oxygen radical, singlet oxygen ($O_2^{\cdot-}$), and
14 hydroxy radical (HO^{\cdot}) are also associated with
15 bactericidal/anti-pathogen activity. In a similar fashion,
16 macrophages taken from BCG-infected animals or otherwise
17 activated have been reported as destroying tumor cells in tissue
18 culture through elaboration of hydrogen peroxide and tumor
19 necrosis factor.

20 C. Affixation element shall mean a cell receptor site
21 moiety such as an antibody or peptide capable of affixing the
22 complex to a site on a cell. The affixation element of the
23 complex is understood to have a binding affinity for the site of
24 cytotoxic environment production. This can be at a site
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1 particular to a tumor, but also particular to certain classes of
2 cells such as interleukin binding sites. An affixation element
3 will also be required to cojoin at least one proöxygenator moiety
4 and preferably more than one such moiety. Examples of affixation
5 elements are the cell binding fragment of diphtheria toxin
6 (fragment B), the IL-2 binding site, and antitumor antibodies
7 such as α -MSH.

8 It is understood that in the practice of this invention,
9 some sites undergo phagocytosis. That is the site of cellular
10 affixation which is initially external becomes drawn into the
11 cell. While it is preferred that the cell bound
12 proöxygenator-affixation element complex remain external to the
13 cell, this is not an absolute requirement. Antibodies are a
14 particular category of affixation element, generally comprising
15 proteins circulating in plasma.

16 D. Complex shall mean a proöxygenator moiety bound to an
17 affixation element such that (1) the proöxygenator moiety remains
18 capable of enzymatically converting an oxygen source substrate
19 into AOS, and (2) the affixation element as complexed to the
20 proöxygenator moiety maintains specificity for the target site of
21 affixation.

22 E. Xanthine oxidase shall mean the the enzyme
23 xanthine:oxygen oxidoreductase, an iron-molybdenum flavoprotein.

1 F. Discrete site cytotoxic environment shall mean the
2 provision of a cytotoxic environment at a defined location
3 proximate to a proöxygenator-affixation element complex bound to
4 a cell, but not limited to the single bound cell.

5 G. Cytotoxic environment shall mean an environment that
6 results in reduction or cessation of proliferation of a cell type
7 and further may include death of some or all cells of a given
8 cell type. Cell is used as an inclusive term encompassing
9 differentiated tissue, single cells, bacteria, multicellular
10 pathogenic organisms, viri, retroviri, and neoplastic cells.

11 Cytotoxic environment shall further be expansively
12 understood to include AOS as a "neo-adjuvant," that is as a
13 potentiator of other therapies. The neo-adjuvant function is
14 displayed in conjunction with other therapy such as radiation,
15 chemotherapy, and vaccine/immunomodulation therapy -- each of
16 which is potentiated by cellular changes including permeability
17 changes and protein expression/recognition changes resulting from
18 the practice of this invention.

19 H. Tumor specific antibody shall mean an antibody that
20 preferentially binds to neoplastic cells. In particular
21 embodiments, antibodies to malignant melanoma, carcinoma,
22 adenocarcinoma, sarcoma (including, Kaposi sarcoma),
23 neuroblastoma, myeloma, lymphoma, and leukæmias.

1 I. Xanthine shall refer to methylxanthines and analogues
2 and derivatives thereof. This shall be understood to include,
3 without limitation, hypoxanthine, caffeine, theophylline,
4 theobromine, dysphylline, enprofylline, and pentoxifylline.

5 J. Therapeutically effective shall mean a dosage that
6 produces the desired physiological effect. As to a
7 proöxygenator-affixation element complex, therapeutically
8 effective means that sufficient complex is bound such that when
9 presented with oxygen bearing substrate a cytotoxic environment
10 arises. In the practice of the method of this invention two
11 steps are required. First the complex must be bound to the
12 target cells in therapeutically effective concentration -- which
13 is necessarily a potential for physiological activity realized as
14 permanent effect only upon the presentation of oxygen bearing
15 substrate. Therapeutically effective as to a dosage of oxygen
16 bearing substrate shall be one sufficient to establish a
17 cytotoxic environment at the site of complex binding in the
18 presence of bound complex. Such dosage provides an environment
19 that results in reduction or cessation of proliferation of a cell
20 type and further may include death of some or all cells of a
21 given cell type or at a given location.

22 In the practice of this invention it will be of importance
23 to select an affixation element that will bind to a target cell
24 in sufficient concentration to ultimately provide therapeutically
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1 effective AOS concentration at the target site. While some
2 pathogens are exquisitely sensitive to AOS others are
3 recalcitrant. Binding of complex at a high density of sites at a
4 high saturation for a lengthy period will be factors tending to
5 increase obtainable AOS levels. Other factors are the number of
6 prooxygenator moieties bound to each antibody, the activity of
7 each prooxygenator moiety, and availability of AOS substrate and
8 the absence of competitive or inhibitory reactants.

9 Tumor Specific Antigens: Tumor cells can frequently be
10 targeted by antigenic determinants. Cells infected with
11 oncogenic viri frequently have two recognition antigens displayed
12 on the cell surface, either of which may provide suitable sites
13 for antibody binding. Oncofetal antigens may be expressed on the
14 surface tumor cells which differentiate adult tissues from tumor
15 tissues. Examples of these are carcino-embryonic antigen (CEA)
16 in cancer of the intestine and α -fetoprotein in hepatic
17 carcinoma. There are available monoclonal antibodies raised
18 against human melanoma cells that also react with tumors of
19 neural origin. Another monoclonal antibody defines the SSEA-1
20 antigen found on a variety of human tumors. Tumors induced by
21 chemical agents such as benzopyrene have tumor specific antigens.
22 Researchers have particularly noted the tumor specificity of the
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1 Ig idiotype on the surface of chronic leukæmic cells. Other
2 tumor specific antigens can be prepared by methods well known in
3 the art and do not comprise a part of this invention.

4 Tumors sensitive to the AOS therapy of this invention, and
5 therapeutically effective dosage levels may be determined by *in*
6 *vitro* techniques which are known in the art. For example, a
7 tumor may be conveniently grown in tissue cultures. To the
8 tissue cultures a variety of proöxygenator-affixation element
9 complexes at a variety of concentrations may be presented with
10 various oxygen source substrates in a checker board assay or the
11 like. The most inhibited tissue cultures will define the
12 therapeutically effective complexes, oxygen source substrates,
13 and may be extrapolated to define a range of therapeutically
14 effective dosages. Additional agents may be cross tested in, for
15 example, traditional *in vitro* Combination Effect Test or the
16 Therapeutic Index Test, to determine if neo-adjuvant activity may
17 be advantageously used as well.

18 The Combination Effect Test employs a series of tests to
19 determined combined drug efficacy. One such test is the "Checker
20 Board Assay" to test different serial dilutions of the drugs to
21 be combined with AOS administration as challenged by a test cell
22 culture of cancer cells in agar or broth. Another test is the
23 Virus Titer Reduction Assay, measuring the reduction in
24 multiplication of virus as grown in host cells. Another test is
25

1 an increase in the therapeutic index which is the dose lethal to
2 50% of the subjects as compared to the dose therapeutically
3 effective in 50% of the cases. The use of the Combination Effect
4 Test allows for the coadministration of AOS with other drugs in a
5 useful and efficacious manner. Particular reference is made to
6 the increased efficacy of Tumor Necrosis Factor by the practice
7 of this invention.

8
9 Complexing a Proöxygenator with an Affixation Element: In
10 combining a proöxygenator moiety with an affixation element care
11 must be taken to preserve the AOS forming activity (usually
12 enzymatic) of the proöxygenator and the binding strength and
13 specificity of the affixation element. To accomplish complexing
14 either chemical or recombinant methods may be usefully employed.
15 As a cojoining methodology, hybridizing IL-2 with a toxin has
16 been described in Greenfield et al., "Science," pp 238, 536
17 (1979). Also, hybridization of diphtheria toxin/IL-2 has been
18 described in U.S. Pat. No. 4,675,382 using recombinant DNA
19 methodologies. Pseudomonas exotoxin A/IL-2 hybridization has
20 been described in Lorberboum-Galski et al., "Proc. Natl. Acad.
21 Sci. USA 85: 1922-26, (1988). The teachings of the foregoing
22 references are incorporated herein by reference. In, for
23 example, Lorberboum-Galski et al., IL-2 replaced the endogenous
24 cell-specific receptor domain of the toxin protein, Pseudomonas
25 exotoxin A/IL-2. Further examples of this technology are set

1 forth in US Patent 5,047,227 to Rodwell, "Novel and Improved
2 Antibodies for Site Specific Attachment of Compounds;" US
3 Patent 4,937,183 to Ultee et al., "Method for Preparation of
4 Antibody-fragment Conjugates;" US Patent 4,867,973 to Goers et
5 al., "Antibody-Therapeutic Agent Conjugates;" and US Patent
6 4,671,958 to Rodwell et al., "Antibody Conjugates for the
7 Delivery of Compounds to Target Sites" the teachings of which are
8 incorporated herein by reference. Similar information is
9 setforth in European Patent Application 90311590.5, Publication
10 No. 425,235 A2, by Chari et al. the teachings of which are
11 incorporated herein by reference.

12
13 The compositions and methods of this invention possess
14 valuable pharmacological properties. The proöxygenator-affixation
15 element complex can localize on or near such targets as tumors
16 cells, cysts, areas of inflammation, and individual viri or
17 retroviri. In the presence of an oxygen source substrate, the
18 proöxygenator-affixation element complex will provide discrete
19 site cytotoxic environment. Such discrete site cytotoxic
20 environment will retard or reverse growth of the target cells or
21 organisms. In some applications the desired effect will further
22 include cytotoxic treatment of other nearby cells or organisms at
23 the same discrete site. The discrete site cytotoxic effect is of
24 great benefit in the field of medicine, particularly in the field
25 of cancer therapy. This benefit is demonstrated, for example,

1 using the method of administering a complex of tumor specific
2 antibody-xanthine oxidase in conjunction with administration of
3 xanthine. A cytotoxic environment at the tumor site is
4 established to preferentially kill tumor cells, with minimal off
5 site toxicity.

6 Thus, these compositions can be used with indications
7 providing a binding site for the complex. Included indications
8 are solid tumor neoplasms as well as systemic neoplasms including
9 cancers, leukemias, viral diseases wherein the virus is
10 "recognized" and attached by the antibody, brucellosis,
11 shistomiasis, malaria, and bacterial infections.

12 The compositions and method are particularly useful as
13 antitumor agents wherein the tumor is strongly antigenically
14 identifiable by the antibody of the complex and wherein the tumor
15 is susceptible to AOS. The composition can be used in
16 conjunction with other therapeutic agents as a neo-adjuvant.

17 In addition, the compositions can be used in *in vitro*
18 diagnostics for determining which target cells are sensitive or
19 susceptible to treatment via AOS (alone or in combination with
20 other drugs) at concentrations obtainable *in vivo*.

21 The compositions of this invention are generally
22 administered to animals, including but not limited to mammals,
23 and avians, and particularly, livestock, household pets, humans,
24 cattle, cats, dogs, poultry, etc.

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1 The pharmacologically active compositions of this invention
2 can be processed in accordance with conventional methods of
3 Galenic pharmacy to produce medicinal agents for administration
4 to patients, e.g., mammals including humans.

5 The compositions of this invention can be employed in
6 admixture with conventional excipients, i.e., pharmaceutically
7 acceptable organic or inorganic carrier substances suitable for
8 parenteral, enteral (e.g., oral or inhalation) or topical
9 application which do not deleteriously react with the active
10 compositions. Suitable pharmaceutically acceptable carriers
11 include but are not limited to water, and salt solutions (e.g.,
12 isotonic saline, buffered saline) and injectable formulations
13 (including i.v., and peritoneal) .

14 The pharmaceutical preparations can be sterilized but must
15 not be denatured. If desired, pharmaceutical preparations may be
16 mixed with auxiliary agents, e.g., lubricants, preservatives,
17 stabilizers, wetting agents, emulsifiers, salts for influencing
18 osmotic pressure, buffers, and the like which do not
19 deleteriously react with the active compositions. They can also
20 be combined where desired with other active agents, e.g.,
21 proöxygenator-affixation element complex administered with an
22 oxygen source substrate.
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1 For parenteral application, particularly suitable are
2 injectable, sterile solutions, preferably aqueous solutions, as
3 well as suspensions, or emulsions. Ampoules are convenient unit
4 dosages. In certain localized administrations the
5 proöxygenator-affixation element complex and/or oxygen source
6 substrate may be administered via intravenous shunt permitting
7 "up stream" introduction of therapeutic agents and "down stream"
8 removal of therapeutic agents. Thus, high localized
9 concentrations of therapeutic agents may be obtained, and yet
10 maintain low systemic levels.

11 Sustained or directed release compositions can be
12 formulated, e.g., liposomes, or those wherein the active
13 component is protected with differentially degradable coatings,
14 e.g., by microencapsulation, multiple coatings, etc. It is also
15 possible in certain applications to freeze-dry the new
16 compositions and use the lyophilates obtained, for example, for
17 the preparation of products for injection.

18 For topical application such as to the lungs, suitable are
19 sprayable aerosol preparations wherein the active ingredient,
20 preferably in combination with a liquid inert carrier material,
21 is packaged in a squeeze bottle or provided by nebulizer.
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1 Intravenous administration is preferred. However, the
2 specific mode of administration will vary with the site of
3 treatment and the particular active agents. The method of
4 administration will preferably be selected to develop the highest
5 AOS concentration at the site of treatment.

6 Dosages of both the proöxygenator-affixation element complex
7 administered and the oxygen source substrate(s) may be determined
8 empirically by methods known to those skilled in the art.

9 However the method and agents of the instant invention are
10 uniquely determinable by calculation. An antibody's affinity for
11 target binding sites is determinable by standard methods.

12 Similarly, the general number of binding sites in a given
13 antibody-receptor application of the invention is determinable.

14 In the case of superoxide (O_2^-) as produced by xanthine
15 oxidase, the following calculations are instructive.

- 16 1. Each xanthine throws off one superoxide, O_2^- .
- 17 2. The specific activity of xanthine oxidase is $\approx 14,000$, thus
18 the enzyme can produce $14,000 \mu M$ of O_2^- per minute.
- 19 3. A given cell has about 40,000 binding sites for a given
20 antibody.
- 21 4. Based on a single cell (and presuming only one enzyme per
22 antibody), the area local to that cell may have 5.6×10^8
23 $\mu M O_2^-$ /min, or roughly 560M/sec.
- 24 5. The lifetime of superoxide is about 10^{-6} to 10^{-9} .

25

1 6. Thus maintained site concentration at an instantaneous
2 sampling is between about 10^{-5} to about 10^{-8} M of
3 superoxide/minute.

4 Concentration levels can be altered by binding more than one
5 enzyme to an antibody, or utilizing enzymes of increased
6 activity. Further, attachment of antibody and associated
7 enzymatic activity as generally distributed in an area will
8 result in nodes of increased AOS concentration.

9 While these will vary widely with each antibody, binding
10 site, volume over which antibody complex is distributed and the
11 half-life of the complex, such determinations are within the
12 recognized skill of practitioners in the art. Dosages based on
13 these factors -- bearing in mind tolerable toxicity levels --
14 will then be determined.

15 In a like fashion, the dosage and time of administration of
16 oxygen source substrate(s) to form AOS from a complex containing
17 xanthine oxidase may be either determined empirically or
18 calculated. In the example of the methylxanthine, caffeine, as
19 an oxygen source substrate(s), the dosage of caffeine will not
20 exceed the capacity of the xanthine oxidase to form AOS.

21 Calculation will include volume throughout which the xanthine is
22 distributed and the half-life of caffeine *in vivo*. In the
23 example of caffeine and theophylline, in humans it is known to be
24 distributed into all body compartments, and its apparent
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1 distribution is about 0.4 to about 0.6 liter/kg of body weight,
2 and higher in premature infants. The half-life of caffeine in
3 plasma is about 3 to 7 hours. Variance in the half-life,
4 however, in specific circumstances is well known to those skilled
5 in the art. For example, the half-life may double in women in
6 the later stages of pregnancy, or be up to 50 hours in premature
7 infants. There is also well document substantial
8 inter-individual variation in clearance of methylxanthines, and
9 such clearance should be tested to determine the individual
10 dosage requirements. Caffeine dosages typically should not
11 exceed 15 mg/kg and plasma concentrations of 30 μ g/ml. Tolerated
12 methylxanthine dosage levels are well known in the art, such as
13 are found in Goodman and Gilman's The Pharmacological Basis of
14 Therapeutics Eighth Edition, Eds., Gilman, Rall, Nies, Taylor
15 (Pergamon Press, New York, New York, 1990), the teachings of
16 which are incorporated herein by reference.

17 The dosage of the compositions according to this invention
18 generally are designed to afford maximal tolerated delivery of
19 AOS to the target site. It will be appreciated that the actual
20 preferred amounts of active compositions in a specific case will
21 vary according to the specific compositions being utilized, the
22 particular compositions formulated, the mode of application, and
23 the particular situs and organism being treated. Dosages for a
24 given host can be determined using conventional considerations,
25

1 e.g., by customary comparison of the differential activities of
2 the subject compositions and of a known agent, e.g., by means of
3 an appropriate, conventional pharmacological protocol.

4
5 In the practice of this invention utilizing xanthine oxidase
6 bound to antibody the proöxygenator-affixation element complex
7 the following steps are taken. A subject in need of AOS
8 therapeutic treatment and having an antibody specific treatment
9 site is administered xanthine to a concentration of about 10^{-9} to
10 about 10^{-5} M. Particular effective concentrations are from about
11 concentration of about 10^{-8} to about 10^{-6} M, as well as from about
12 concentration of about 10^{-6} to about 10^{-5} M. If toxicity is at
13 issue, maximum concentration is established over time, with
14 xanthine administration curtailed when unsuitable toxicity begins
15 to be manifested. Maximum concentration is reached about 1 hour
16 after oral administration. In a 70kg subject, administration of
17 xanthine in doses of from about 300 mg to 500mg is useful.
18 Thereafter the proöxygenator-affixation element complex, xanthine
19 oxidase bound to an antibody specific to the treatment site,
20 administered intravenously to establish a concentration which
21 will bind to binding sites in from about 20% to 100% of such
22 sites. Xanthine oxidase bound to said antibody is periodically
23 readministered in proportion to the rate at which enzyme-antibody
24 is deactivated, here about every three hours. Due to the long
25 half-life of xanthine, it is not usually necessary to

1 readminister xanthine during the course of this treatment. In
2 particular embodiments it is useful to administer the
3 proöxygenator-affixation element complex prior to administration
4 of the substrate.

5 Example 1

6 Xanthine Oxidase/ α -MSH Complex

7 To a human suffering from malignant melanoma, xanthine is
8 administered intravenously to obtain a plasma level of 10-30 μ g/ml
9 which is maintained over 4 hours by additional xanthine
10 administration as required. Twenty minutes after initial
11 xanthine administration, a proöxygenator-affixation element
12 complex consisting of a proöxygenator moiety of xanthine oxidase
13 and an affixation element of α -melanocyte stimulating hormone
14 (α -MSH) is administered, i.v. The xanthine oxidase/ (α -MSH
15 complex is suspended in isotonic saline. Administration is
16 intravenous at a dosage of 100 mg every ten minutes until 80% of
17 the binding sites on target cells are occupied. As used herein
18 binding cites on target cells refers to the binding of the
19 proöxygenator-affixation element complex the at the site of
20 cytotoxic environment production. Such binding results from the
21 affinity between complex and binding cite. This treatment is
22 repeated daily for 5 days.

I claim:

1. A proöxygenator-affixation element complex.
2. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises a proöxygenator moiety of at least one enzyme.
3. The complex of Claim 2 wherein the enzyme is a xanthine oxidase.
4. The complex of Claim 2 wherein the enzyme is a superoxide dismutase.
5. The complex of Claim 2 wherein the enzyme is a myeloperoxidase.
6. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises an affixation element being an antibody.
7. The complex of claim 6 wherein the antibody binds to melanoma, carcinoma, adenocarcinoma, sarcoma, neuroblastoma, myeloma, lymphoma, or leukemia cells.

8. The complex of Claim 6 wherein antibody is α -MSH, carcino-embryonic antigen, α -fetoprotein, or SSEA-1.
9. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises an affixation element being an peptide.
10. The complex of claim 9 wherein the peptide is the diphtheria fragment B, or IL-2 binding site.
11. A method of producing discrete site cytotoxic environment in an animal, including a human, comprising the steps of
administering to said animal a therapeutically effective dosage of a proöxygenator-affixation element complex wherein said complex has a binding affinity for the site of cytotoxic environment production; and thereafter,
administering to said animal a therapeutically effective amount of an oxygen source substrate;
forming an activated oxygen species (collectively, "AOS").
12. The method of Claim 11 wherein upon administration said affixation element of the proöxygenator-affixation element complex performs the step of
binding the complex to a cell.

13. The method of Claim 11 wherein the proöxygenator element comprises xanthine oxidase, superoxide dismutase, or myeloperoxidase.
14. The method of Claim 11 wherein the oxygen source substrate is a methylxanthine.
15. The method of Claim 14 wherein the methylxanthine is xanthine.
16. The method of Claim 14 wherein the methylxanthine is caffeine.
17. The method of Claim 14 wherein the methylxanthine is theophylline.
18. The method of Claim 11 further comprising the step of maintaining the AOS concentration in a discrete area to at least about 10^{-8} M/minute for a particular intervals of at least about 15 minutes.

19. The method of Claim 18 further comprising the step of maintaining the AOS concentration in a discrete area to at least about 10^{-6} M/minute for a particular intervals of at least about 15 minutes.

20. The method of Claim 19 further comprising the step of maintaining the AOS concentration in a discrete area to at least about 10^{-5} M/minute for a particular intervals of at least about 15 minutes.

21. A method of Claim 11 wherein
administering to said animal a therapeutically effective dosage of a proöxygenator-affixation element complex comprises binding said complex to at least about 50% of the binding sites at said site of cytotoxic environment production.

22. The method of Claim 21 wherein said binding is to at least about 80%.

23. A method of diagnosing AOS treatable tumors comprising:
adding to a tissue culture of a tumor to be tested two or more graduated dosages of a proöxygenator-affixation element complex wherein said complex has a binding affinity for the tumor being tested; and thereafter,

administering to said culture a therapeutically effective
amount of an oxygen source substrate;
determining tumor growth inhibition in said tissue culture.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04582**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : A61K 45/05, 39/00, 37/48, 37/62; C12N 9/02
US CL : 424/94.2, 94.1, 94.3, 85.1, 85.2, 85.91; 435/189
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.2, 94.1, 94.3, 85.1, 85.2, 85.91; 435/189; 514/410, 185

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, Medline, Biosis, Registry

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,906,469 (Jansen, et al.) 06 March 1990, see entire document, especially col. 1, lines 26-31, col. 5, lines 11-12, col.6, lines 6-8 and 54-64, and col. 13, lines 6-9.	1-23
Y	MOLECULAR AND CELLULAR BIOCHEMISTRY, Vol. 10(1), issued 31 January 1976, A. Bozzi, et al., "Enzyme Defense Against Reactive Oxygen Derivatives. II. Erythrocytes and Tumor Cells," pages 11-16, especially pages 11 and 12.	1-23
Y	US, A, 4,971,991 (Umemura, et al.) 20 November 1990, see entire document.	1-23
Y	US, A, 4,975,278 (Senter, et al.) 04 December 1990, see entire document.	1-23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/04582

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,762,707 (Jansen, et al.) 09 August 1988, see entire document.	1-23
A	US, A, 4,937,183 (Ultee, et al.) 26 June 1990.	1-23
A	US, A, 4,671,958 (Rodwell, et al.) 09 June 1987.	1-23
A	US, A, 4,867,973 (Goers, et al.) 19 September 1989.	1-23
A	ACCOUNTS OF CHEMICAL RESEARCH, Vol. 5(10), issued October 1972, I. Fridovich, "Superoxide Radical and Superoxide Dismutase," pages 321-326.	1-23

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